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Evaluation of Antimicrobial Potential of Endophytic Fungi and GC-MS Metabolic Profiling of *Cephalosporium* sp., and *Fusarium moniliforme*

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Abstract

From last few decades, microbes gained special attention residing inside plant tissues, now called endophyte. Studies proved that these microbes are able to produce biologically active metabolites and effective candidates against various pathogens. Endophytic fungi are a source of natural therapeutic products. Therefore, endophytic *Fusarium* species are isolated from different plants. These endophytic *Fusarium* species showed potential against common laboratory bacteria (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium* and *Bacillus subtilis)*. *Fusarium moniliforme* and *Cephalosporium* sp., were further selected to isolate compounds by GC-MS techniques. The mycelial extract of *Cephalosporium* sp., and oily fraction of filtrates of *F. moniliforme* revealed the presence of several therapeutic compounds based on the peak areas, molecular weights, Rt (retention times) and m/z (mass fragmentations). The major bioactive metabolites identified from these fungi are Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-pivalate, Salicylamide, 1,2-Benzenedicarboxylic acid, Geranyl isovalerate, and diisooctyl ester, and reported to possess antibacterial, antioxidant, nematicidal, and antifungal potential. These results will lead to further in-depth research into the potential cause of plants endophytes interactions. The wide application of fungal origin bio products offers an effective prospect for discovering novel therapeutic agents in order to combat infectious agents as well as agricultural pests.

Keywords: Fungal isolates, Antibacterial potential, Culture filtrates, GC-MS analysis

Introduction

From last three decades, the term endophytes have appeared frequently in mycological literature. Though, original term of endophytes can be traced back from the 19th century and their definition is totally opposite from their original one. The use of the term is not always consistent nor as they are accepted by all researchers [1]. Such a term is being used for those fungi that have been symptomless on healthy host plant tissues. The word endophytic fungi are used for those fungi colonizing in plant tissues causing no immediate effects on host plant. Survey of fungal diversity of various hosts from last two decades has highlighted endophytic colonization in land plants [2]. Blackwell [3] reported the identification, characterization and fungal discovery, and estimated the broad range of their diversity [4] to global estimates of 5.1 million based on sequencing methods. Researchers have estimated endophytic fungal species to be about 1 million [5]. Several studies identified that the members of Ascomycetes are adapted to a specific ecosystem of different range [6].

Although, endophytic fungi are described by researchers for a longer time as they gain much more attention. Most of the isolates of fungi are considered to be a source of novel products possessing wide application and may be used in medicine which is initially isolated from higher plants [7]. Fungal based bioactive metabolites isolated from endophytes offer a unique chance for discovering a new therapeutic drug against various maladies.

Initially it is considered that the genus *Fusarium* are responsible for plant destruction and are pathogens of food and produces mycotoxins that have great concern to food products and their safety. *Fusarium* species are pathogenic to most of the economically important crops and produce structurally diverse bioactive compounds that have adverse health effects [8,9]. Endophytic fungi may have extraordinary biodiversity and may provide a large untapped opportunity to isolate bioactive metabolites with unique chemical structure. The recent advancement in ST (Screening Technology) revealed the diversity of fungal endophytes for biomolecules and their potential role in medicinal and agricultural fields [10]. Due to the presence of these promising active metabolites, plants are able to combat stress conditions whether it is biotic or abiotic stress. Endophytic fungi produce a nature friendly compound that has no negative role and is eco-friendly. Most of the agricultural products are synthesized synthetically which are toxic to the environment as well as to plants. Therefore, for nature safety, synthetic agricultural products will be replaced with fungal based products. Novel bioactive metabolites having fungal origin may be better to eradicate the infectious agents and agriculture pests [11].

In other ways, there is much more to gain by harnessing the metabolic products of fungal endophytes. Some novel antibiotics, biofuels, anticancer agents, and antioxidants are identified from endophytic fungi. It is proved from studies that the main operative inhibitory classes of biologically active metabolites are isoamyl acetate and esters [12]. It is considered that antimicrobial bioactive metabolites, antimicrobial biomolecules, and anti-insectal secondary metabolites are mostly of fungal origin. The fungal based volatile group of compounds may separate and be characterized by a variety of chemical profiles and techniques [10]. Another fungal species *Daldinia concentrica*, was found in olive plants in the form of endophytic fungus and are a source of bioactive metabolites that are effective to treat pre and postharvest diseases in plants [13]. The major role of these volatile bioactive compounds is to prevent fruits from rotting and destruction. However, they also protect grapes against *Botrytis cinerea,* tomato fruit and orange paste against *Penicillium digitatum,* and peanuts against *Aspergillus niger*. In the agriculture industry, synthetic products of promising volatile compounds have great application. Genomic characterization of plant endophytes indicated that endophytic fungi have gene clusters encoding volatile compounds, in which most of them are unable to express under standard-laboratory conditions. To adapt to selective environmental conditions endophytic fungi express their volatile compounds [14]. This report focuses on the description and isolation of endophytic fungal isolates and their potential role against common laboratory bacteria. This study elaborates the GC-MS (Gas Chromatography- Mass Spectrometry) based metabolic profiling of metabolites from mycelial extract of *Cephalosporium* sp.*,* and culture filtrate of *Fusarium moniliforme*.

Materials and Methods

Samples

In this study, different healthy wild and cultivated plant samples (with the hypothesis that these harbors a unique microbial community) were collected from the agricultural area of Karachi, Malir, and Karachi University for the isolation of endophytic fungi. The plants sample were brought to the laboratory and endophytic fungi were isolated from leaves, stem, and roots. The area of sample collection had sandy loam soil with pH 8.0 and enough nutrients were presents as farmers apply on regular basis to such areas for the plant's growth and development. The fungi were isolated from the samples within 24 h.

Isolation and identification of endophytic fungi

The plant samples (1g, each) were thoroughly washed with tap H_2O, sterilized with (1%) bleach washed with 70% alcohol for about 3 min, then rinsed with distilled water. In 50 mL sterilized water, plant samples were chopped in sterilized grinder and dilutions were made for each sample up to $1:10^4$. The suspension (0.1 mL) from the final dilution was transferred onto Potato Dextrose Agar (PDA) media in Petri plates which were supplemented with penicillin (100,000 units/liter) and streptomycin (0.2 g/liter). The isolated fungi were then purified on PDA media after incubation at 25-30°C for 1 week. Fungi showed strong *in vitro* activity were initially identified through morphological characters and further confirmed by molecular techniques. For molecular identification, fungal mycelium (1.5–2 cm^2) was obtained from the fresh culture in sterile 1.5 mL micro centrifuge tubes, and the cells were disrupted in liquid nitrogen using glass beads. DNA was isolated using Biobasic EZ-10 Spin Column, Fungal Genomic DNA Mini-Preps Kit, (CANADA). The quality of isolated DNA was evaluated by 1% agarose gel electrophoresis. The rDNA region was amplified by using primer ITS1-5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3'. The Polymerase Chain Reaction (PCR) was carried out on ABI 2700 thermal cycler (California, USA) using the following conditions: first hold for 5 min at 94°C followed by 35 cycles; denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, and after that final extension at 72°C for 5 min and the hold the reaction at 4°C. The amplified PCR products were evaluated by using 2% agarose gel electrophoresis. The amplified products were submitted to BGI Genomic Services

(Shenzhen, Guangdong, China) for Sanger sequencing of both ITS1 and ITS4 primers [15].

Endophytic fungal culture filtrates

The Czapek's Dox broth (300 mL) was used to grow endophytic fungi in 500 mL conical flasks, each having a fungal disc of 5 mm. The incubation period was 2 weeks at 25-30°C. After incubation period, selective broths were filtered, and the filtrates exposed to chloroform vapors to completely remove any sort of propagule. The fungal culture filtrates were further used for characterization of secondary metabolites [16].

Antibacterial activity of culture filtrates

For antibacterial activity, culture broth was used. 5 mm sterile discs were used to impregnate having filtrates at (20, 40, and 60 [mL/disc]) and impregnated discs were then dried in sterile chambers. The bacterial lawn of particular bacteria *viz., (S. aureus, P. aeruginosa, B. subtilis, E. coli* and *S. typhimurium*) prepared on TSA (Trypticase Soy Agar). All the culture filtrates loaded discs (20, 40 and 60 [mL/disc]) kept at the periphery of the plates and penicillin (20 mg/disc) used as +ve control and sterile broth considered as -ve control. The TSA plates incubated at room temperature for 24 hours and their zone is measured [16].

Fractions of culture filtrates of *Fusarium moniliforme*

To isolate compound from *Fusarium moniliforme,* culture infiltrates of *Fusarium moniliforme* were utilized, and *n-hexane* was employed to extract its metabolites. *n*-hexane residue part was then extracted with chloroform solvent and finally both concentrated on rotary evaporator until all solvent was evaporated. 1.5 mg/mL dilution was prepared from *n*-hexane concentrated fraction in a required solvent for antibacterial activity. 5 mm thick filter paper disc loaded with dilutions and then dried. Finally, the disc was placed on nutrient Agar plates and after 24 hours their zone of inhibition was measured [17].

Metabolites from mycelial extract of *Cephalosporium sp.,*

The mycelium (10 gm) of *Cephalosporium* sp., initially immersed in *n*-hexane solvent to completely remove excessive H_2O. For the isolation of metabolites, the mycelium was extracted in *n*-hexane solvent using volume up to 200 mL by Soxhlet apparatus. Finally, all the solvent from fraction is evaporated by a rotary vacuum evaporator (40°C). *n*-hexane fraction was isolated after solvent evaporation and the final concentration of *n*-hexane fraction was oily [18,19].

Gas Chromatography-Mass Spectrometry (GC-MS)

Oily *n*-hexane fraction of culture filtrates and mycelial extract was subjected to GC-MS analysis for the characterization of metabolites. GC-MS conducted on Agilent (6890) Gas Chromatograph equipped with ZB-SMS (30 m x 0.32ID) and 0.25 um thick film column which was hyphenated with MS (mass spectrometer), Jeol, JMS- 600 H, operational mode was EI managing ion source at 50°C. E.E (Electron energy) was maintained at (70 eV) and carrier gas volume was maintained in between 1 to 5 mL. Individual peaks of compounds were assigned by comparing their RI (retention indices) and MS (mass spectra) by computer matching against NIST (National Institute of Standards and Technology) USA, MSDC (Mass Spectrometry Data Center) [20].

Analyzing data

For antibacterial activity, software SPSS were used for the data analysis and their significant value was calculated at $P<0.05$. Values in column bearing same superscript letter are not significantly different at $p<0.05$ according to Duncan's multiple range test. [21].

Results

Fungal isolates

The identified isolates are *Fusarium moniliforme, Fusarium solani* and *Cephalosporium* sp., (KUCC13885). After the identification of fungi, their pure forms are deposited in the Karachi University Culture Collection Center. The accession number were issued from University Culture Collection Center, where such study was conducted for fungal isolates; *Fusarium solani* (KUCC-1362, KUCC-1363, KUCC-1368, KUCC-1372, KUCC-1373, KUCC-1379, KUCC-1381), *Cephalosporium* sp., (KUCC-13885), and *F. monilforme* (KUCC-13884) (**Table 1**).

Antibacterial activity of fungal culture filtrates

Fungal isolates were tested against gram +ve and gram -ve bacteria and showed potential at 60 mL/disc. At different concentrations of culture filtrates, fungi showed different ranges of inhibition rate. Whereas, against Gram negative bacteria such as *Salmonella typhimurium, Pseudomonas aeruginosa*, and *E. coli* at 60µl/disc produced more than 10 mm zone of inhibition, while less than 10 mm zone was measured against *Bacillus subtilis* and *Staphylococcus aureus* at 60 µl/disc (**Table 1**).

Antibacterial activity of fractions of fungal culture filtrates

Both fractions of culture filtrates showed different ranges of inhibition at different concentrations against gram +ve and -ve bacteria. The slight variation is observed in both fractions even at the same concentration. 10 mm inhibition zone is measured by *n*-hexane soluble fraction for *S. aureus, P. aeruginosa, S. typhimurium, B. subtilis,* and *E. coli* at 60 mL/ disc of concentration. The chloroform soluble fraction showed an inhibition zone more or less 10 mm at 60 mL/ disc against *S. aureus, P. aeruginosa, S. typhimurium, B. subtilis,* and *E. coli* (**Table 2**).

Table 1. *In vitro* growth inhibition of *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium, Bacillus subtilis* and *Escherichia coli* by culture filtrates of endophytic *Fusariu*m spp.

Fungi isolated	**Name of host**	***S. aureus***	***P. aeruginosa***	***S. typhimurium***	***B. subtilis***	***E. coli***
Control		$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$
Fusarium moniliforme (+ve)	*Helianthus annuus* L.	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$0^{a} \pm 0.0$	$7^{cd} \pm 1.8$	$7^{bc} \pm 1.8$	$0^{a} \pm 0.0$	$9^{bc} \pm 2.9$
40 µL/disc		$11^{de} \pm 2.1$	$13^{de} \pm 3.1$	$9^{ef} \pm 2.9$	$7^{cd} \pm 1.8$	$12^{cd} \pm 1.8$
60 µL/disc		$16^{f} \pm 2.4$	$18^{k} \pm 2.5$	$15^{j} \pm 2.0$	$12^{f} \pm 1.8$	$17^{g} \pm 2.5$
F. solani (+ve)	*Helianthus annuus* L.	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$0^{a} \pm 0.0$	$8^{bc} \pm 1.8$	$9^{bc} \pm 2.9$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$
40 µL/disc		$7^{b} \pm 1.8$	$9^{gh} \pm 2.9$	$11^{d} \pm 2.1$	$7^{b} \pm 1.8$	$7^{bc} \pm 1.8$
60 µL/disc		$11^{de} \pm 2.1$	$14^{i} \pm 2.2$	$16^{gh} \pm 2.4$	$10^{bc} \pm 2.0$	$13^{d} \pm 3.1$
F. solani (+ve)	*Atriplex stocksii* Boiss	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$7^{b} \pm 1.8$	$8^{bc} \pm 1.8$	$7^{c} \pm 1.8$	$0^{a} \pm 0.0$	$7^{b} \pm 1.8$
40 µL/disc		$12^{cd} \pm 1.8$	$16^{de} \pm 2.4$	$14^{d} \pm 2.2$	$7^{b} \pm 1.8$	$10^{bc} \pm 2.0$
60 µL/disc		$14^{e} \pm 2.2$	$19^{g} \pm 4.0$	$17^{de} \pm 2.5$	$10^{bc} \pm 2.0$	$14^{de} \pm 2.2$
F. solani (+ve)	*Chenopodium album* L.	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$0^{a} \pm 0.0$	$7^{b} \pm 1.8$	$8^{bc} \pm 1.8$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$
40 µL/disc		$7^{bc} \pm 1.8$	$9^{bc} \pm 2.9$	$10^{d} \pm 2.0$	$8^{b} \pm 1.8$	$10^{b} \pm 2.0$
60 µL/disc		$10^{e} \pm 2.0$	$14^{gh} \pm 2.2$	$12^{e} \pm 1.8$	$12^{cd} \pm 1.8$	$17^{f} \pm 2.5$
F. solani (+ve)	*Catharanthus roseus* (L.) G. D	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$0^{a} \pm 0.0$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$	0a ±	$8^{b} \pm 1.8$
40 µL/disc		$9^{bc} \pm 2.9$	$8^{c} \pm 1.8$	$10^{de} \pm 2.0$	$0^{a} \pm 0.0$	$10^{bc} \pm 2.0$
60 µL/disc		$11^{e} \pm 2.1$	$16^{gh} \pm 2.4$	$12^{f} \pm 1.8$	$7^{b} \pm 1.8$	$13^{d} \pm 3.1$
F. solani (+ve)	*Corchorus olitorius* L.	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$0^{a} \pm 0.0$	$7^{bc} \pm 1.8$	$7^{b} \pm 1.8$	$0^{a} \pm 0.0$	$7^{b} \pm 1.8$
40 µL/disc		$7^{b} \pm 1.8$	$8^{e} \pm 1.8$	$11^{bc} \pm 2.1$	$8^{b} \pm 1.8$	$10^{cd} \pm 2.0$
60 µL/disc		$11^{cd} \pm 2.1$	$17^{fg} \pm 2.5$	$15^{e} \pm 2.0$	$10^{bc} \pm 2.0$	$19^{de} \pm 4.0$
F. solani (+ve)	*Achyranthus aspera* L.	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$	$7^{b} \pm 1.8$	$0^{a} \pm 0.0$	$8^{b} \pm 1.8$
40 µL/disc		$12^{bc} \pm 1.8$	$15^{bc} \pm 2.0$	$11^{cd} \pm 2.1$	$8^{b} \pm 1.8$	$10^{cd} \pm 2.0$
60 µL/disc		$16^{hi} \pm 2.4$	$17^{d} \pm 2.5$	$19^{gh} \pm 4.0$	$15^{cd} \pm 2.0$	$19^{ij} \pm 4.0$
F. solani (+ve)	*Chenopodium album* L.	$8^{bc} \pm 1.8$	$11^{bc} \pm 2.1$	$13^{de} \pm 3.1$	$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$
20 µL/disc		$7^{b} \pm 1.8$	$9^{bc} \pm 2.9$	$7^{b} \pm 1.8$	$0^{a} \pm 0.0$	$9^{c} \pm 2.9$
40 µL/disc		$12^{cd} \pm 1.8$	$14^{d} \pm 2.2$	$13^{c} \pm 3.1$	$8^{d} \pm 1.8$	$10^{d} \pm 2.0$
60 µL/disc		$16^{i} \pm 2.4$	$19^{f} \pm 4.0$	$15^{cd} \pm 2.0$	$14^{ef} \pm 2.2$	$11^{de} \pm 2.1$

Values in column bearing same superscript letter are not significantly different at $p<0.05$ according to Duncan's multiple range test.
*+ve Control (Penicillin 20 mg/disc).

Table 2. *In vitro* antibacterial activity of *n*-hexane and chloroform fractions of culture filtrates of endophytic fungi.

Endophytic Fungi	*S. aureus*		*P. aeruginosa*		*S. typhimurium*		*B. subtilis*		*E. coli*	
	Hexane	Chloroform	Hexane	Chloroform	Hexane	Chloroform	Hexane	Chloroform	Hexane	Chloroform
Control	0	0	0	0	0	0	0	0	0	0
+ve control*	12	10	13	7	11	9	8	7	7	5
Fusarium solani										
20 µL/disc	8	5	10	7	8	6	6	0	11	0
40 µL/disc	10	8	13	11	10	7	9	5	13	7
60 µL/disc	12	9	16	13	16	12	11	9	15	9
Fusarium moniliforme										
20 µL/disc	9	8	11	8	9	8	8	5	11	6
40 µL/disc	11	9	13	10	11	11	11	6	14	9
60 µL/disc	13	10	17	15	17	14	13	10	18	11
*+ve Control (Penicillin 20 mg/disc).										

Compounds characterization

In vitro study *Fusarium moniliforme* (KUCC-13884) and *Cephalosporium sp.* (KUCC-13885) showed potential against common laboratory bacteria- *S. aureus, P. aeruginosa, S. typhimurium, B. subtilis,* and *E. coli* rather than the rest of the endophytic fungi. So, the culture filtrates of *F. moniliforme* (KUCC-13884) and mycelial extract of *Cephalosporium sp.* (KUCC-13885) are further selected for the characterization of compounds by GC-MS.

GC-MS profiling of metabolites from culture filtrate of *Fusarium moniliforme*

Hexane soluble metabolites were extracted from culture filtrate of *Fusarium moniliforme* by GC-MS. The compounds reported included Salicylamide, *n*-Hexadecanoic acid, 2-ethylhexyl ester, 1-Heptatriacotanol, 3, 5-dehydro-6-methoxy-pivalate, 2, 2, 4- Trimethyl-3 (3, 8, 12, 16-tetramethylheptadeca 3, 7, 11, 15-tetraenyl)-cyclohexanol, Geranyl isovalerate, Heptacosane, 2-Propenoic acid, 3-(4-methoxy-phenyl)-, 1,2-Benzenedicarboxylic acid, diisooctylester-, Cholest-22-ene-21-ol, and Hexadecanoic acid, octadecyl ester (**Table 3**; **Figures 1-3**; **Supplementary Table S1**).

Table 3. Tentatively Identified Constituents from *n*-hexane soluble fraction of culture filtrate of endophytic fungus *Fusarium moniliforme.*

Peak #	Systemic name	Molecular Formula	Mol.wt.	t_R (min)	Conc.
1.	Salicylamide	$C_7H_7NO_2$	137	18.55	0.1
2.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	29.34	0.79
3.	2-Propenoic acid, 3-(4-methoxy-phenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	290	48.42	0.11
4.	1-Heptatriacotanol	$C_{37}H_{76}O$	536	49.18	0.11
5.	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy- pivalate-,	$C_{33}H_{54}O_3$	498	50.21	0.13
6.	Geranyl isovalerate	$C_{15}H_{26}O_2$	238	51.92	0.08
7.	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	52.66	0.11
8.	Heptacosane	$C_{27}H_{56}$	380	53.37	0.11
9.	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy- pivalate-	$C_{33}H_{54}O_3$	498	56.17	0.09
10.	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetra -enyl)-cyclohexanol	$C_{30}H_{52}O$	428	56.89	0.14
11.	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-pivalate-,	$C_{33}H_{54}O_3$	498	63.36	0.23
12.	Hexadecanoic acid, octadecyl ester	$C_{34}H_{68}O_2$	508	64.53	0.15

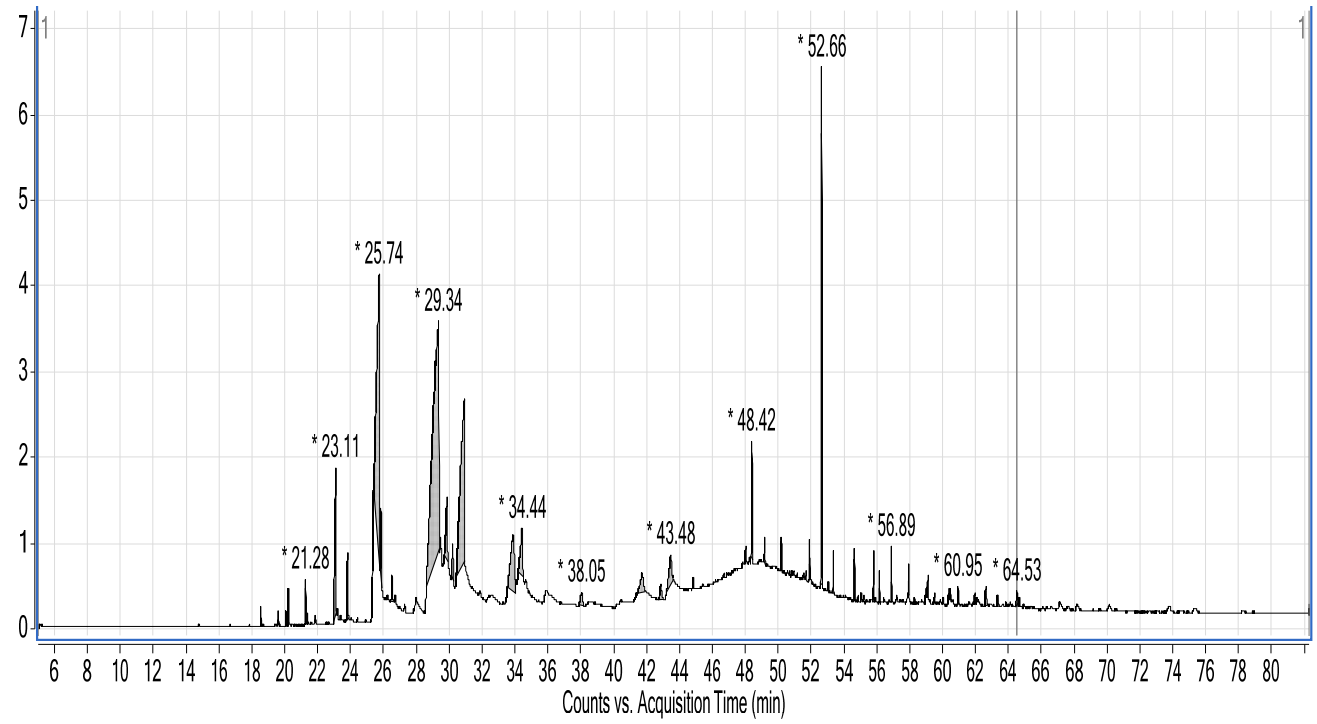


Figure 1. GC Chromatogram showing different compounds present in *n*-hexane soluble fraction of culture filtrate of *Fusarium moniliforme*.

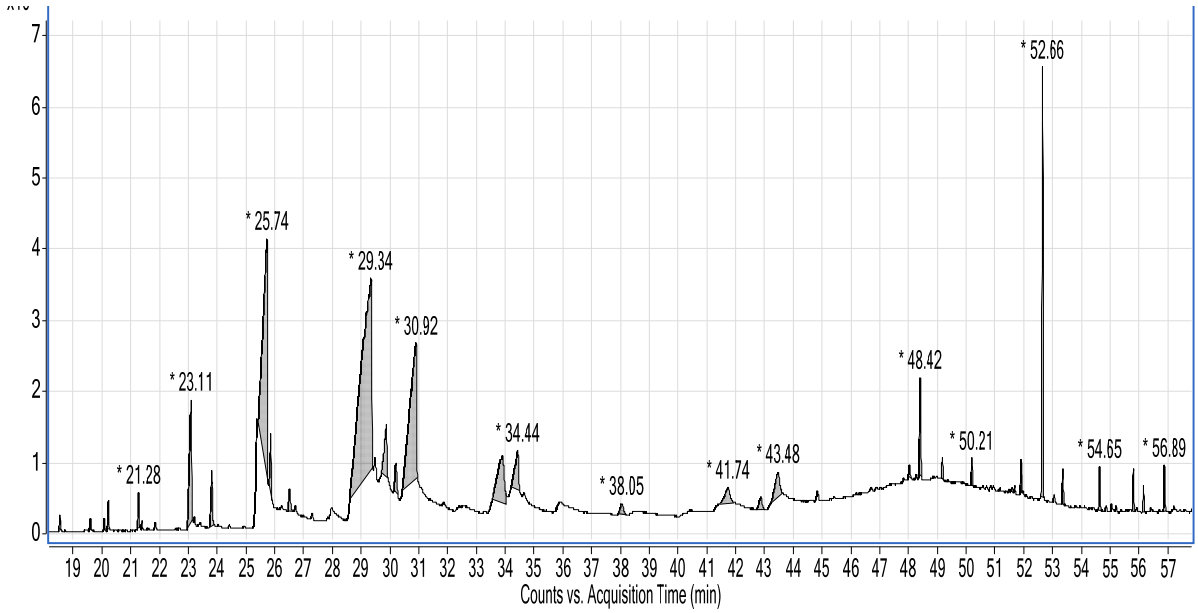
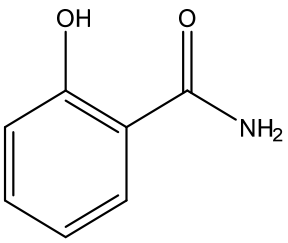
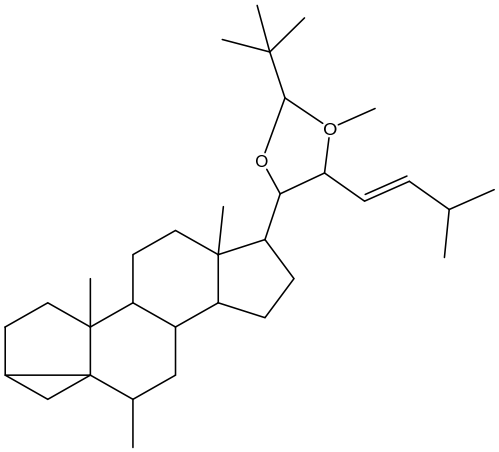


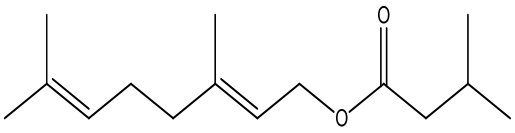
Figure 2. GC Chromatogram showing different compounds present in *n*-hexane soluble fraction of culture filtrate of *Fusarium moniliforme*.



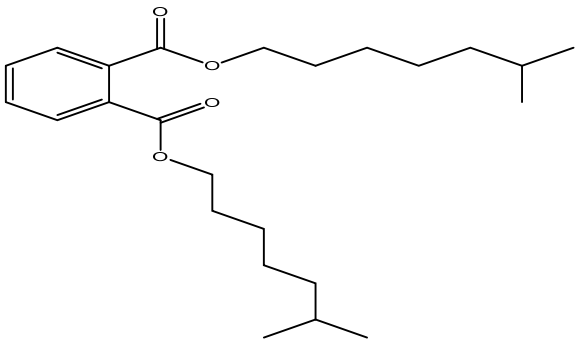
Salicylamide



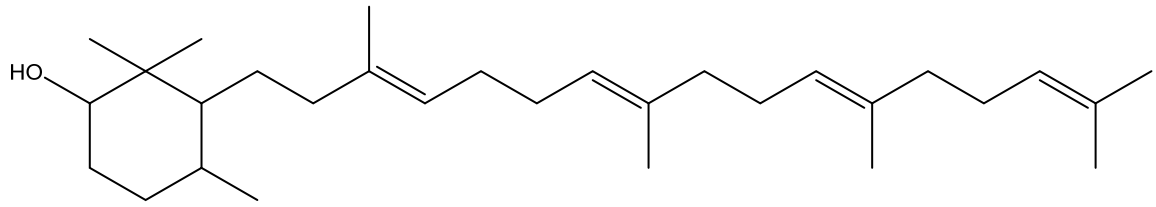
Cholest-22-ene-21-ol,-3,5-dehydro-6-methoxy-pivalate



Geranyl isovalerate



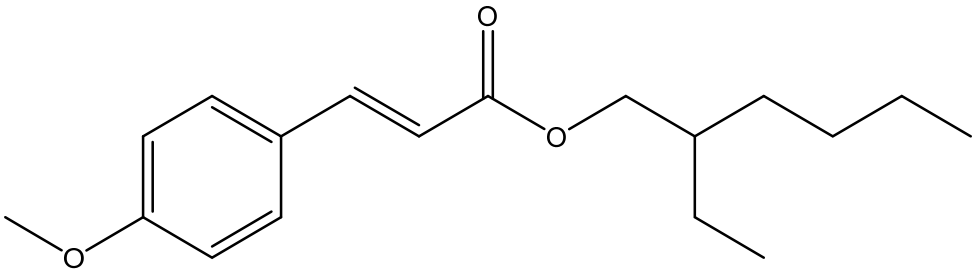
1,2-Benzenedicarboxylic acid, diisooctyleste



2,2,4-trimethyl-3-((3*E*,7*E*,11*E*)-3,8,12,16-tetramethylheptadeca-3,7,11,15-tetraen-1-yl)cyclohexan-1-ol

Hexadecanoic acid, octadecyl ester

***n*-Hexadecanoic aci**



2-ethylhexyl (*E*)-3-(4-methoxyphenyl)acrylate

Figure 3. New compounds tentatively identified from *n*-hexane soluble fraction of culture filtrate of *Fusarium moniliforme.*

GC-MS profiling of metabolites from endophytic fungus *Cephalosporium sp.*

Mycelial extract *Cephalosporium* sp. was also used for the characterization of metabolites by GC-MS. A total of twenty three new compounds were characterized from this source. These included (Z) -1-(3-methyl-5,6-dihydropyrazin-2-yl) hex-1-en-2-ol, 4-(1-hydroxy-2-isopropyl-5-methylcyclohexyl) but-3-yn-one, 1,5,7,8-Tetrahydro-2,6-pteridinedione, 3-Methyl-ene-1-oxa-spiro-[4,5]-decan-2-one, methyl-8-(2-hexyl-[1,1-bi(cyclopropane)]-2yl)octanoate, 9-Octadecenoic acid, 9,12-Octadecadienoic acid, cis-Vaccenic acid, Ethyl 9.cis.,11.trans.-octadecadienoate, Oleic Acid, methyl 4-(2-((2-((2-((2-pentylcyclopropyl)methyl)cyclopropyl)methyl) cyclopropyl)butanoate 9,12,15-Octadecatrienoic acid-2,3-di-hydroxypropyl ester, 7-Methyl-tetradecen-1-ol acetate, 1,2-Benzenedicarboxylic acid, diisooctyl ester, Octadecane-3-ethyl-5-(2-ethylbutyl), 2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetra-enyl)-cyclohexanol, Oleic acid, 3-(octadecyloxy)propyl ester, 17-(5,6-dimethylhept-3-en-2-yl)-10,13-dimethyl-2,3,4,10,12,13,14,15,16,17-decahydro-1H1-cyclopenta[a]phenanthrene-3-yl-4 methylbenzenesulfonate -Heptatriacotanol, 9-Octadecenoic acid-2-hydroxy-1-(hydroxymethyl)-ethyl ester and Ergosta-4,6,8-(14)-22-tetra-en-3-one-, Ethyl iso-allocholate (**Table 4, Figures 4-7, Supplementary Table S-2**).

Table 4. Tentatively Identified Constituents of endophytic *Cephalosporium* sp. obtained from *n*-hexane soluble fraction of mycelium.

Peak#	Systemic name	Molecular Formula	Mol.wt.	t_R (min)	Conc.
1.	Menthol, 1-(butyn-3-one-1-yl), (1S,2S,5R)-	$C_{14}H_{22}O_2$	222	18.72	0.08
2.	2-(2-Hydroxy-hex-1-enyl)-3-methyl-5,6-dihydropyrazine	$C_{11}H_{18}N_2O$	194	20.41	0.25
3.	1,5,7,8-Tetrahydro-2,6-pteridinedione	$C_6H_6N_4O_2$	166	22.25	0.68
4.	3-Methylene-1-oxa-spiro- [4,5]-decan-2-one	$C_{10}H_{14}O_2$	166	22.47	0.17
5.	9-Octadecenoic acid-(Z)	$C_{19}H_{36}O_2$	296	25.05	0.17
6.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	30.28	0.46
7.	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl	$C_{21}H_{38}O_2$	322	37.44	0.25
8.	9,12-Octadecadienoic acid (Z,Z)-,	$C_{18}H_{32}O_2$	280	41.09	0.94
9.	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	41.7	0.51
10.	Ethyl 9.cis.,11.trans.-octadecadienoate	$C_{20}H_{36}O_2$	308	42.74	0.25
11.	Oleic Acid	$C_{18}H_{34}O_2$	282	42.13	0.32
12.	Cyclopropanebutanoic acid, 2-[[2- [[2-[(2-pentylcyclopropyl)-methyl] -cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester	$C_{25}H_{42}O_2$	374	46.64	0.13
13.	1-Heptatriacotanol	$C_{37}H_{76}O$	536	50.17	0.08
14.	9,12,15-Octadeca-tri-enoic acid, 2,3dihydroxy propyl ester, (Z,Z,Z)-	$C_{21}H_{36}O_4$	352	50.58	0.1
15.	Oleic Acid	$C_{18}H_{34}O_2$	282	50.68	0.13
16.	7-Methyl-(Z)-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268	51.89	0.11
17.	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	52.62	0.1
18.	Octadecanal, 2-bromo-	$C_{18}H_{35}BrO$	346	53.34	0.09
19.	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366	54.63	0.15
20.	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetra-enyl)-cyclohexanol	$C_{30}H_{52}O$	428	56.15	0.11
21.	Oleic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$	592	56.88	0.1
22.	9(11)-Dehydroergosterol tosylate	$C_{35}H_{48}O_3S$	548	57.67	0.12
23.	Ergosta-4,6,8-(14),22-tetra-en-3-one-,	$C_{28}H_{40}O$	392	63.85	0.17
24.	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	70.07	0.27
25.	9-Octadecenoic acid-(Z)-, 2-hydroxy-1-(hydroxymethyl)-ethyl ester	$C_{21}H_{40}O_4$	356	74.44	0.4

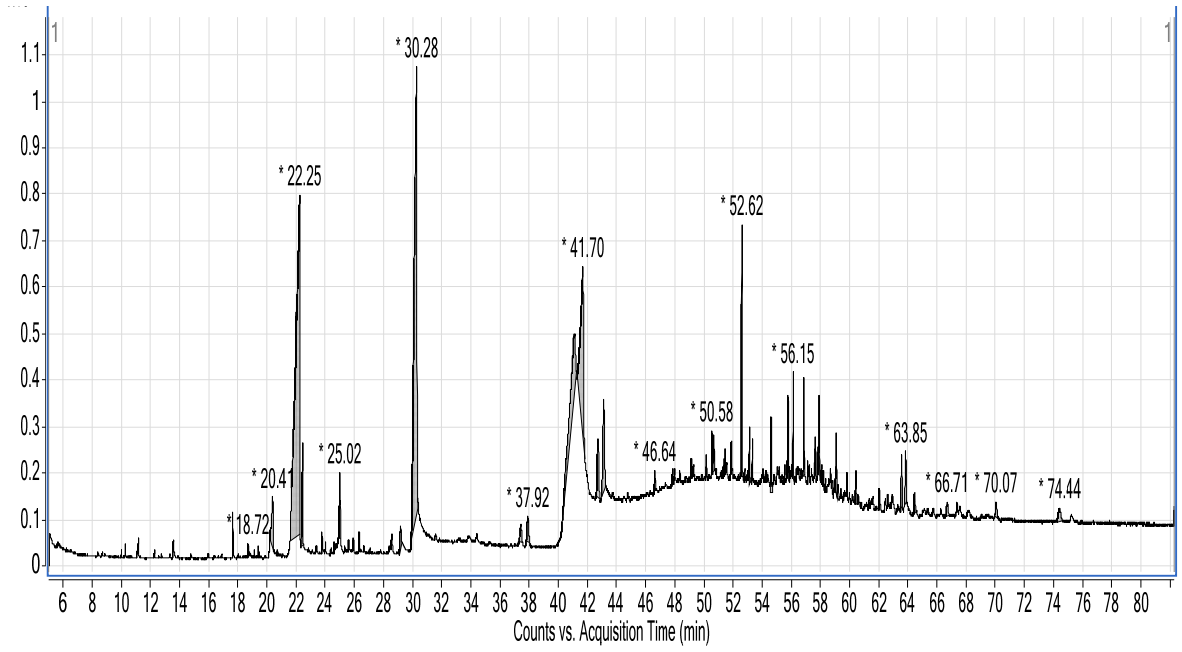


Figure 4. GC Chromatogram showing different compounds present in *n*-hexane soluble fraction of mycelium of *Cephalosporium* sp.

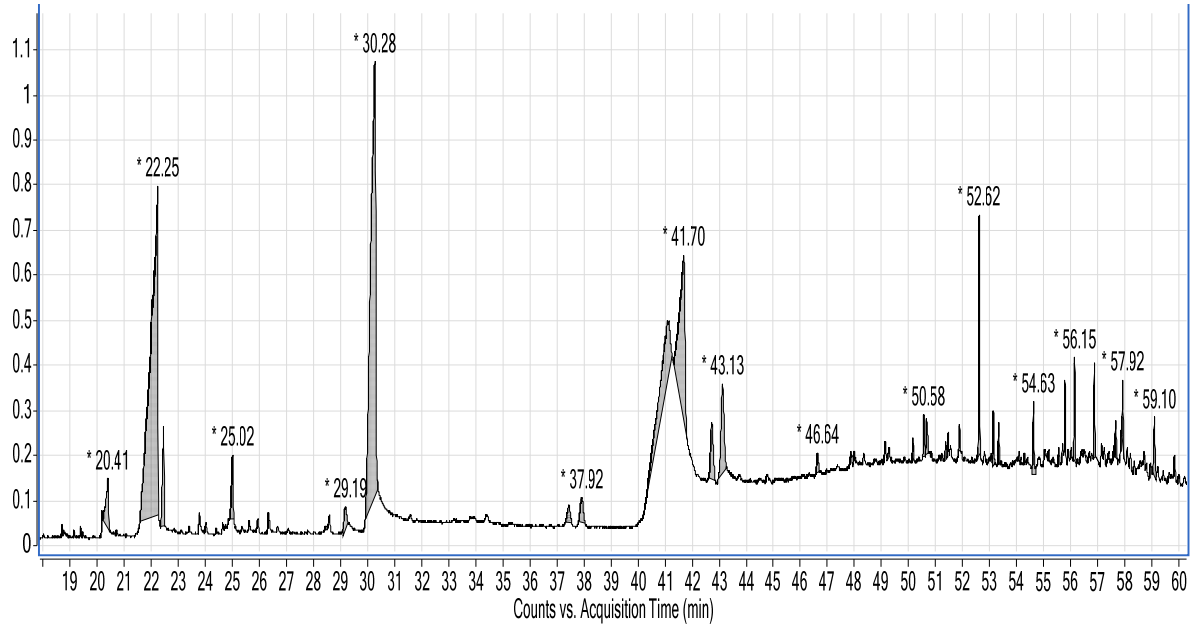


Figure 5. GC Chromatogram showing different compounds present in *n*-hexane soluble fraction of mycelium of *Cephalosporium* sp.

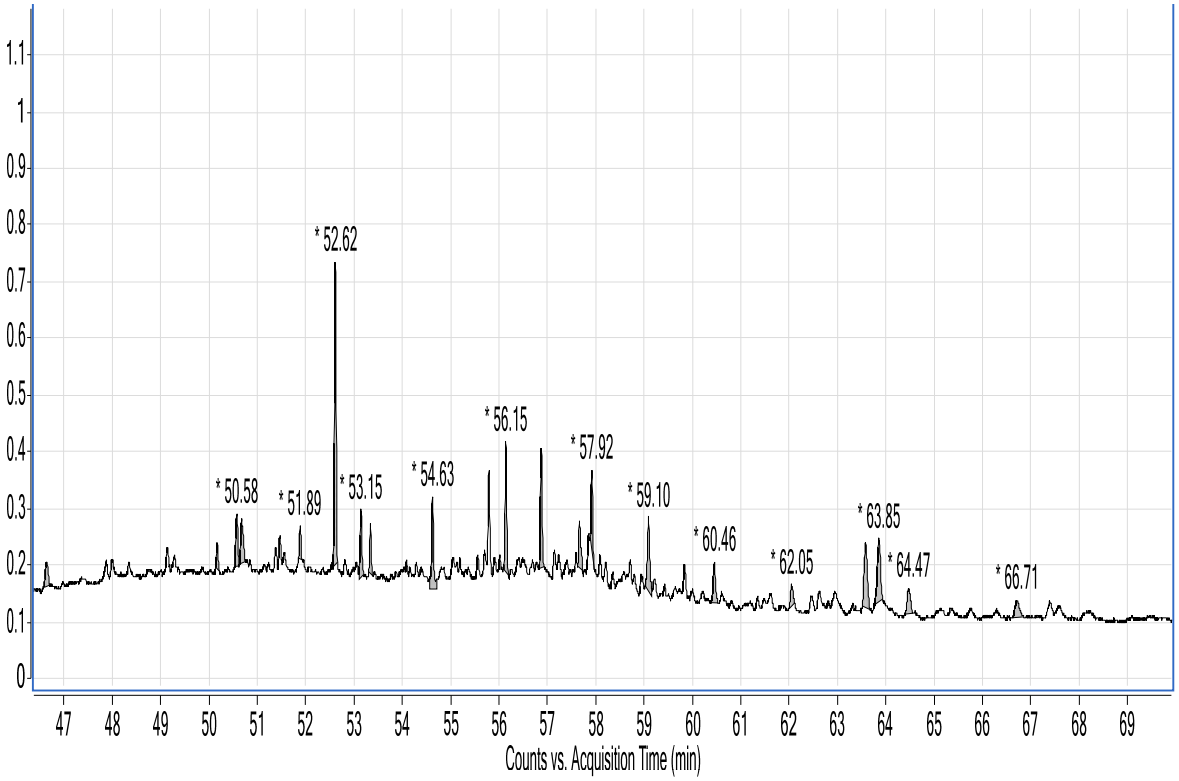
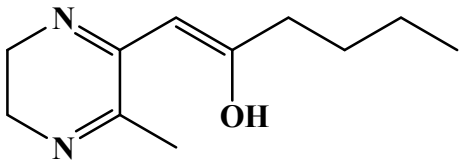
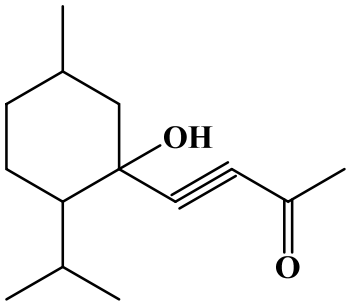


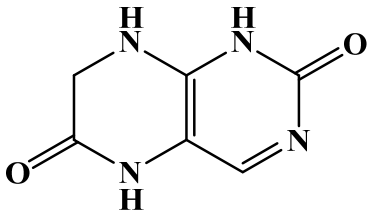
Figure 6. GC Chromatogram showing different compounds present in *n*-hexane soluble fraction of mycelium of *Cephalosporium* sp.



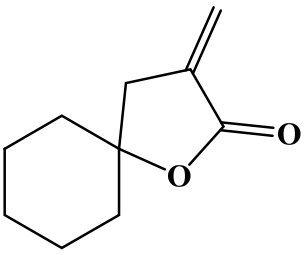
(*Z*)-1-(3-methyl-5,6-dihydropyrazin-2-yl)hex-1-en-2-ol



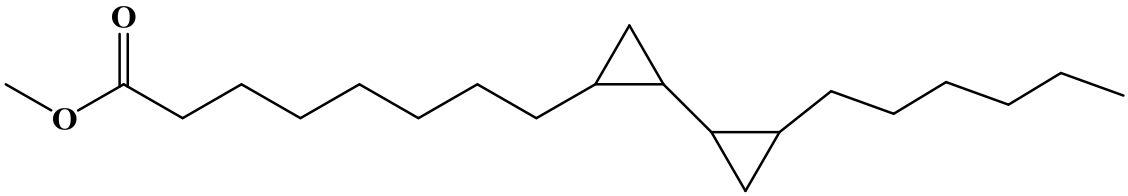
4-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)but-3-yn-2-one



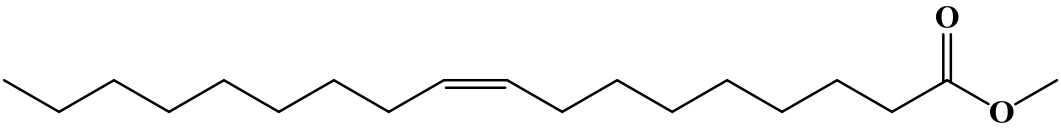
1,5,7,8-tetrahydropteridine-2,6-dione



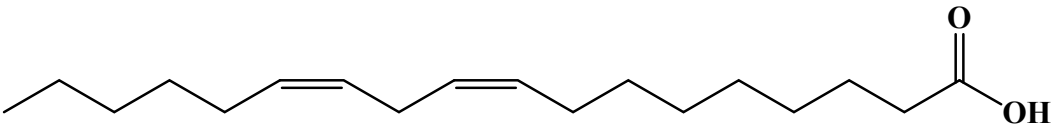
3-methylene-1-oxaspiro[4.5]decan-2-one



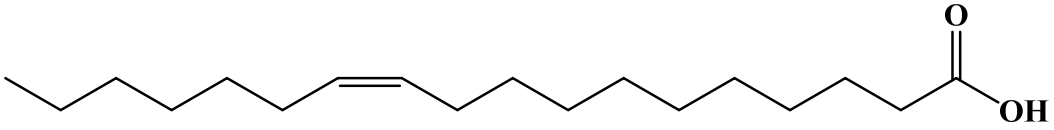
methyl 8-(2'-hexyl-[1,1'-bi(cyclopropan)]-2-yl)octanoate



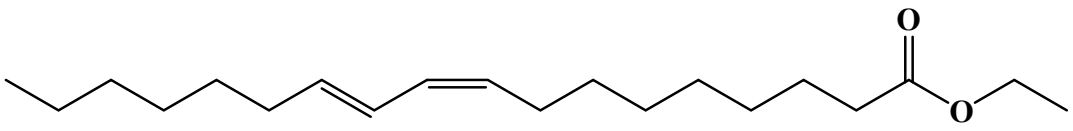
9-octadecenoic acid (Z)-, methyl ester



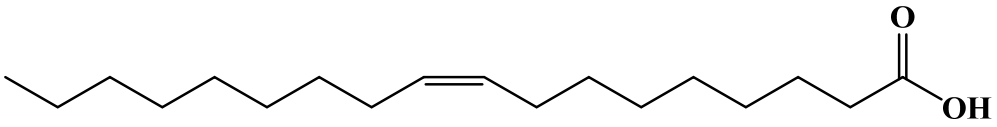
9,12-octadecadienoic acid (Z,Z)-



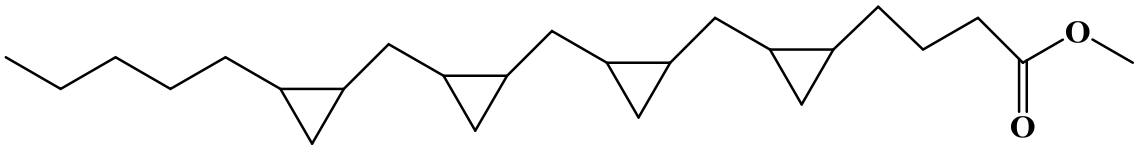
cis-vaccenic acid



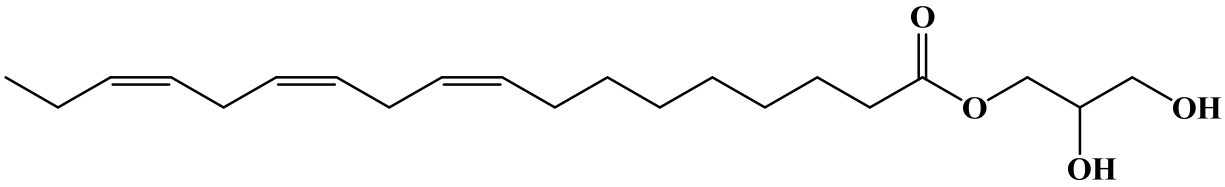
Ethyl 9.cis.,11.trans,-octadecadienoate



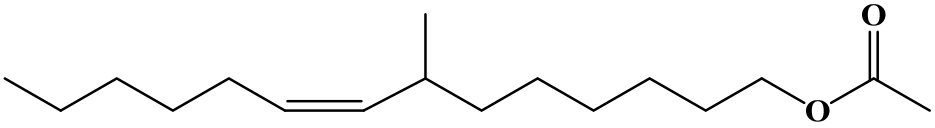
Oleic Acid



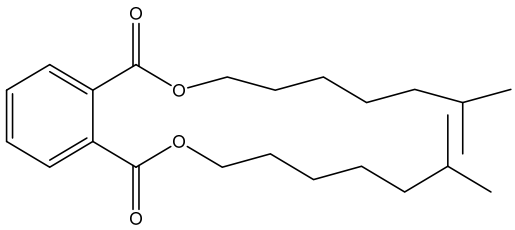
methyl 4-(2-((2-((2-((2-pentylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)butanoate



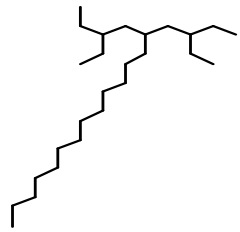
9,12,15-Octadecatrienoic acid-2,3-di-hydroxypropyl ester, (Z,Z,Z)



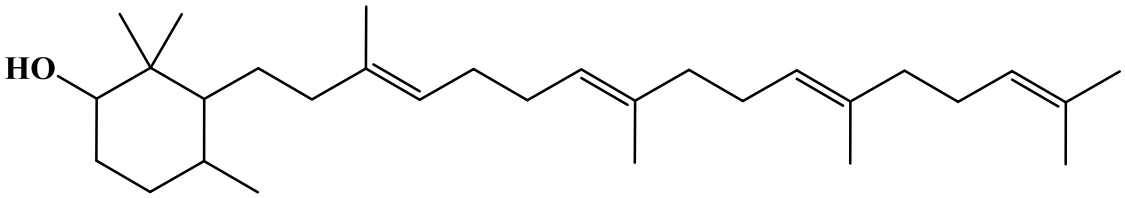
(*Z*)-7-methyltetradec-8-en-1-yl acetate



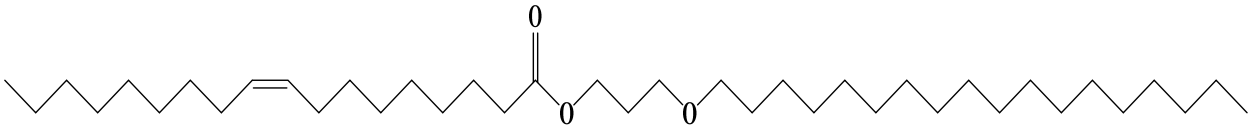
1,2-Benzenedicarboxylic acid, diisooctyl ester



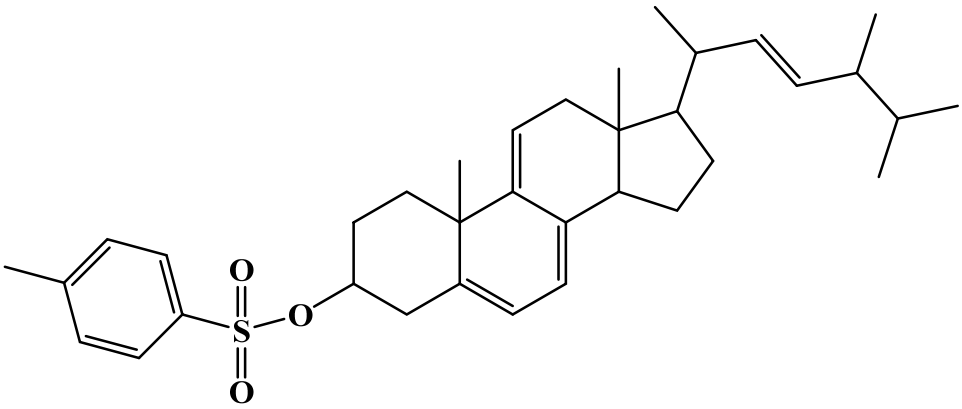
Octadecane-3-ethyl-5-(2-ethylbutyl)



2,2,4-trimethyl-3-(3,8,12,16-teramethyl-heptadeca-3,7,11,15-tetra-enyl)-cyclohexanol

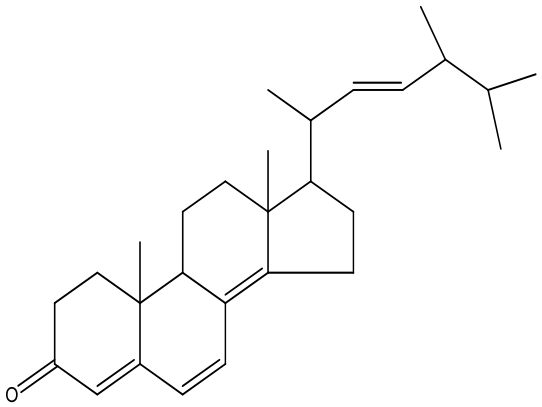


oleic acid, 3-(octadecyloxy)propyl ester

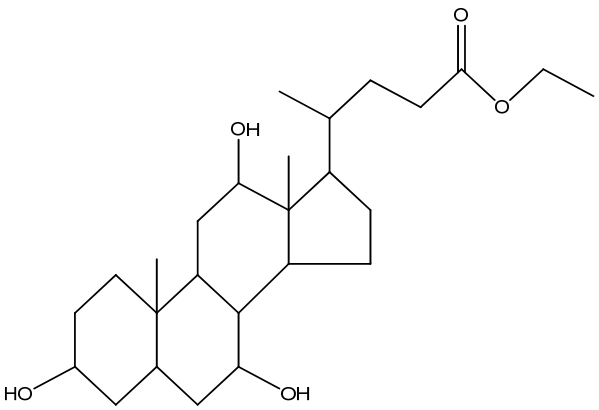


(*E*)-17-(5,6-dimethylhept-3-en-2-yl)-10,13-dimethyl-2,3,4,10,12,13,14,15,16,17-decahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl 4-methylbenzenesulfonate

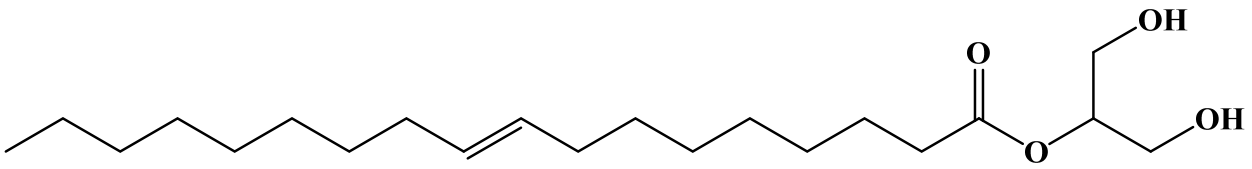
heptatriacontan-1-ol



Ergosta-4,6,8-(14)-22-tetra-en-3-one-,



Ethyl iso-allocholate



9-octadecenoic acid-2-hydroxy-1-(hydroxymethyl)-ethyl ester

Figure 7. New compounds identified from *n*-hexane soluble fraction of mycelium of *Cephalosporium* sp.

Discussion

The term endophytes are used for those microbes that reside within plant organs and are found in every plant species and share different ecological places. Fungal endophytes are a wide source of secondary compounds, other new products also isolated from these organisms with promising application and widely used in drug development and pharmaceutical products. Bérdy [22] identified approximately 20,000 active secondary bioactive compounds that are of fungal origin. Fungi are placed among the diverse groups of eukaryotes which are recognized for novel metabolites production that directly act as drugs. The drug discovery or in other words the success story of fungal derived by-product used as a drug was initiated from the isolation of first antibiotic drug penicillin obtained from *Penicillium* sp., antifungal product griseofulvin from *P. griseofulvum,* cyclosporine from *Cylindrocarpon lucidum*, lovastatin from *Aspergillus terreus* and many more fungal taxa twisted the view of drug researchers from medicinal plants to endophytic fungi [23]. Recently, the crucial role of fungal bioactive metabolites in the development of novel pharmaceutical drugs is increasing, studies are also under progress to use endophytes as plant growth promoters and to reduce plant diseases [17].

In current study, isolated fungal strain was initially identified by using taxonomic keys and literature, and then identification was confirmed by molecular technique like PCR (Polymerization Chain Reaction) amplification of rDNA, ITS regions are transcribed, and their Sanger sequencing results were published. The culture filtrates of fungal isolates are tested for antibacterial activity (*in vitro* activity). The culture filtrates of both fractions (*n*-hexane and chloroform) of *Fusarium* spp., are also tested against pathogenic bacteria. Endophytic fungi exhibited strong potential for antimicrobial activity *in vitro* are further used for the isolation of metabolites by GC-MS profiling. In this study endophytic fungi like *Fusarium solani,* and *Fusarium moniliforme* isolated belongs to phylum Ascomycota. Endophytic fungi are a complex and diverse group of fungi, mostly polyphyletic, belonging to ascomycetes while others are anamorphic fungi [24]. Approximately 300,000 plant species exist in the biosphere and endophytes are host specific to one or more plants. It is illustrated that endophytic fungal taxa are a hyper diverse group of fungi as they are counted as one million in number [11]. Most of the bioactive secondary compounds act as a defensive weapon for the protection of host plants from disease as these secondary metabolites have specific interactions with host plants [25]. Habitat adapted symbiosis specified by Rodriguez et al. [26] illustrated that fungi tolerate stress in a specific manner under certain natural conditions. Mechanisms involved behind this habitat may result in response to plant tolerance against high stress levels are still not known.

Fungal endophytes are the main sources of bioactive metabolites, mostly are considered to be a part of increasing the adaptation of fungi and their broad spectrum to stress environment. In our previous study secondary metabolites were isolated from *Fusarium solani* by GC-MS analysis. The fungal based product possesses strong biological potential against different pathogenic fungi as well as bacteria. The nematicidal metabolites isolated from fungal endophytes are highly diverse in their chemical structure, ranging from simple fatty acids and diverse organic acids to lactones, alkaloids, peptaibiotics, derivatives of hybrid and anthraquinones. In this study the metabolites isolated from fungal endophytes are the long chain aliphatic and aromatic compounds that make them able for antibacterial activity. These compounds have already been reported as having antioxidant, antifungal, anti-insecticidal activity in literature. Strobel et al. [12] reported antibacterial activity of five groups of volatile compounds which includes lipids, esters, ketones, and alcohol from different fungi [27]. They may promote the growth of their host plant by utilizing their nutritional elements, mostly N (Nitrogen) and P (phosphorus) [28]. It is conferred that fungal endophytes that are mutualistic to plants contribute extensively to plant adaptation to stress environment by elevating their tolerance level to drought or to extreme temperature [24]. Tolerance of plants to stress may be due to specific symbionts responsible for the better establishment of plants to high stress environment [26]. Plants face certain environmental challenges in the form of salt stress, cold, drought, or pathogens attack and develop strategies to overcome it. Certain challenges may be observed in plants in response to biochemical changes like phytoalexin production, cellular necrosis, and hypersensitive response. Due to long term evolution, two main resistance types are formed against pathogens like specific resistance and non-specific resistance [29]. The defense mechanism of plants may increase rapidly as they make associations with endophytes by producing secondary metabolites. Endophytes may affect virtually all types of relationship like plant to pathogen, plant to plant, and plant to herbivore interaction [30]. Strobel [31] suggested that fungal endophytes residing within plant tissues have the ability to secrete a certain range of compounds which may be similar too or sometimes with higher activity rate to their selective host. Isolated endophytic fungal isolates *Cephalosporium sp.,* and *Fusarium moniliforme* showed strong antimicrobial activity. This study also focused on the characterization of metabolites which are effective candidates for boosting antimicrobial activity. Findings suggested fungal isolates possess novel metabolites and may be introduced in different sectors of drug industries.

Conclusions

Studies suggested that fungal endophytes emerged as a major form of therapeutically active metabolites which could be an alternate source for agriculture and pharmaceutical industries. Therefore, fungal endophytes that grow in various

ecological niches can be isolated, identified and their active metabolites should be characterized. The active compounds isolated from such domains may further be assessed for their biological purpose and they may gain the attention of researchers in future.

Abbreviations

GC-MS: Gas Chromatography and Mass Spectrometry; Rt: Retention times; m/z: Mass fragmentations; PDA: Potato Dextrose Agar; ITS: Internal Transcribed Spacer; PCR: Polymerase Chain Reaction; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information; TSA: Trypticase Soy Agar; NIST: National Institute of Standards and Technology; MSDC: Mass Spectrometry Data Center

Authors' Contributions

HF and FU carried out the collection of plants, isolation and identification of endophytic fungi and conducted experiments. HF and NS prepared the figures and tables. SU did the antibacterial activity of endophytic fungi. GC-MS analysis was done by MS and MA. HF conceived and designed the study, supervised research work, and improved the quality of the final version of manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgment

The help of Prof. Dr Syed Ehtesham-ul- Haq (now late), Department of Botany, University of Karachi, Pakistan, for the support of research work is highly acknowledged. Support of Prof. Dr. Viqar Uddin Ahmad (now late) in GC-MS profiling is acknowledged.

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